INVESTIGATIONS OF HEAT SHOCK PROTEIN EXPRESSION IN THE SWORDTAIL FISHES *Xiphophorus birchmanni* AND *X. malinche*

A Senior Scholars Thesis

by

ASHLEY MICHELLE MEADERS

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Investigations of Heat Shock Protein Expression in the Swordtail Fishes *Xiphophorus birchmanni* and *X. malinche* (April 2008)

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Research Advisor: Dr. Gil G. Rosenthal
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In many species, animals must endure environmental stressors such as exposure to heat, toxins, and ultraviolet radiation. To protect cells against such stressors, animals employ an evolutionarily ancient mechanism: the heat shock response. During a heat shock response, heat shock proteins (Hsps) act as "chaperones," assisting in maintaining the appropriate conformation of other critical proteins which would otherwise be denatured. Samples from three populations of swordtail fishes were collected from the Sierra Madre Oriental in Mexico to study the relationship between the amount of natural thermal stress and the quantity of Hsps expressed. In each population, HSP70 and HSP90 were quantified in fish taken directly from the natural environment and fish that underwent an acute heat stress experiment. In both cases, the population that experienced very little environmental heat stress expressed significantly less Hsps than the population that experienced much greater natural thermal variation. If climate temperatures within the Sierra Madre Oriental significantly rise, the swordtail population with the least expression of Hsps will have a significantly lower chance at survival than the swordtail population with the highest expression of Hsps.

DEDICATION

To my loving family, whose support, encouragement and wisdom have made me who I am today.

ACKNOWLEDGMENTS

I am grateful to Dr. Rosenthal and Dr. Coleman for the opportunity to pursue this research project. This amazing experience would not have been possible without the support and guidance provided by both gentlemen.

I would like to extend my gratitude to my family for their patience and encouragement. A special thanks to my parents for teaching me how to explore the world and to see the beauty it holds within.

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CHAPTER I

INTRODUCTION

Many animals experience daily temperature fluctuations extreme enough to require specific adaptations to protect the animal's cells from damage. Of the wide variety of thermal tolerance mechanisms animals have developed, heat shock proteins (Hsps) are one of the most widely and heavily researched. Hsps are molecular chaperones that aid in the folding of essential proteins within cells when activated by stress. Although Hsps can be induced by many different types of stress, thermal stress is the most frequently studied. Research suggests a positive relationship between the induction of thermal stress and the production of Hsps (Fader *et al*. 1994). When an acute fluctuation of heat is experienced by an organism, cellular proteins that are in the folding process or have begun to denature are recognized and protected by the Hsps which act as chaperones to provide a stable environment for the proteins to fold properly (Feder & Hofmann 1999). This suggests the more Hsps expressed within a cell, the more efficient the cell will be at surviving and recovering from acute thermal fluctuations (Fader *et al.* 1994). However, there is also a cellular cost to maintain a large amount of Hsps since the proteins are large chaperones, requiring excessive amounts of ATP to be spent in their production (Tomanek & Somero 1999). This energy expenditure would be wasteful if the organism does not experience excessive stress, therefore not needing a large amount of Hsps.

This thesis follows the style of *Proceedings of the Royal Society B: Biological Sciences.*

Within the Sierra Madre Oriental in northeastern Mexico flows a river that connects five distinct populations of *Xiphophorus*, or swordtail fish (Rosenthal & Ryan 2001). The swordtail species *Xiphophorus malinche* and *X. birchmanni* are ideal for evaluating the evolutionary and ecological dynamics of inducible molecular defenses. *X. birchmanni* is broadly distributed over lowland areas (elevation 161-300 m) of the southern Río Pánuco drainage of the Atlantic slope of central Mexico, while *X. malinche* are restricted to highland tributaries (658-1499 m). Hybrid populations are found at intermediate elevations (272-1188 m). The best-characterized hybrid zone, the Río Calnali effectively represents an upstream to downstream gradient of distinct hybrid populations, each subjected to a unique combination of environmental stressors. The geographic position of each population creates distinct ecological variation in the type and intensity of environmental stress that individuals experience, providing researchers with an ideal situation for studying the influence environmental stress has on the evolutionary ecology of these swordtail populations.

X. malinche at Chicayotla live in swift moving, clear spring water shaded by dense overhead canopy keeping both daily and seasonal temperatures consistent and low. *X. malinche* encounter very little thermal stress as a result of the lack of thermal fluctuation within these conditions. The hybrids in the Rio Calnali are located at a wider point along the river where less shade is available to keep the water temperature low and stable. *X. birchmanni* at Garces reside in the slowest, widest, and warmest portion of the river. Sparse canopy coverage at Garces also allows the daily water temperatures to fluctuate about ten degrees, the greatest daily variance of any population.

The stepwise variety of thermal stress found among these populations provides insight into how biological mechanisms have evolved specific adaptations to the demands of a given environment. The understanding of how Hsps respond to environmental changes in temperature creates intrigue about the variation of *Hsps* expression between populations and species. The natural environment of *X. malinche*, *X. birchmanni* and their hybrids along the Sierra Madre Oriental raises many questions about how these fish express *Hsps* as a result of their particular environment. Each population experiences daily and seasonal thermal fluctuations distinct from the other populations leading researchers to believe there will be an equally distinct variation in the expression of *Hsps*. For example, *X. malinche* at Chicayotla typically experience a daily temperature variation of only a few degrees while *X. birchmanni* in Garces can combat daily thermal variance of at least ten degrees. The more drastically the temperature fluctuates, the more equipped an organism must be to combat the resulting thermal stress. By this reasoning, when subjected to acute thermal stress, *X. birchmanni* should express the largest amount of *Hsps*, *X. malinche* expressing a significantly smaller amount, and the Calnali hybrids expression level falling between the two extremes.

The methods of testing the above hypothesis involve (1) logging daily temperature changes to find the amount of thermal variation experienced at both daily and seasonal levels, (2) collecting individuals from each population to examine the quantity of *Hsps* expressed when subjected to acute thermal stress, (3) compare the resulting quantitative differences in Hsps expression between the three populations in relation to natural thermal stress. The remainder of this thesis will examine on a more fundamental level the methods, results, discussion and conclusions developed to test this hypothesis.

CHAPTER II

METHODS

To measure daily temperature fluctuations, HOBO temperature loggers (Onset Corporation) were placed under the water at Garces and Chicayotla. These loggers recorded the water's temperature every fifteen minutes from June 30, 2007 through December 22, 2007. The resulting data was the calculated to configure the average temperature experienced within each population.

At each location, fish were collected using baited traps. Immediately after collection, individuals were sacrificed and placed in RNAlater (Ambion, Inc.). RNA was isolated from brain tissue using a TRIzol reagent (Invitrogen) isolation. Superscript III First-Strand Synthesis System (Invitrogen) was used to create cDNA. Hsp qPCR primers were designed using Primer3 primer design software (http://frodo.wi.mit.edu/) based on *Poecilia reticulata hsp*70 coding sequence (GenBank accession number: AB298594) and *Danio rerio hsp*90 coding sequence (GenBank accession number: NM_131328); *hsp*70 forward primer: TCAGCCAGAACAAGAGAGCA; reverse primer: CAATCTCAATGCTTGCCTGA; *hsp*90 forward primer: GGTGGACTCTGAGGATCTGC; reverse primer: CGATGGGCTCGATCATGTAG. qPCR was conducted using Sybr Green (Invitrogen) chemistry at the Center for Functional Genomics, University of Albany, Albany, NY, USA. For analysis, a single reference sample was designated in the first run, and the same sample was used in each subsequent run; for each sample QuantumRNA Universal 18s RNA (Ambion, Inc.) was used as an internal control.

The next stage of research involved an acute heat shock experiment that was performed on four *X. birchmanni* and four *X. malinche* individuals. All eight individuals had been kept in laboratory tanks at approximately 23 degrees Celsius for 14 months prior to the acute experiment. The acute heat shock experiment involved placing a single fish in an Erlenmeyer flask filled with 1000 mL of water at 23 degrees Celsius. The flask was then placed over a Bunsen burner set to heat the water at a rate of ten degrees in ten minutes. Timing began the instant the flask was placed above the flame until the fish lost equilibrium, at which point the flask was removed from heat. The fish was immediately removed from the flask, sacrificed, and the head was placed in RNAlater. Then, the RNA isolation / reverse transcription / qPCR procedure described above was repeated

ANOVA was used to investigate population differences in mean Hsp expression patterns. If an ANOVA revealed significant (P<0.05) differences, then Fisher LSD tests were used to investigate differences among specific means.

CHAPTER III

RESULTS

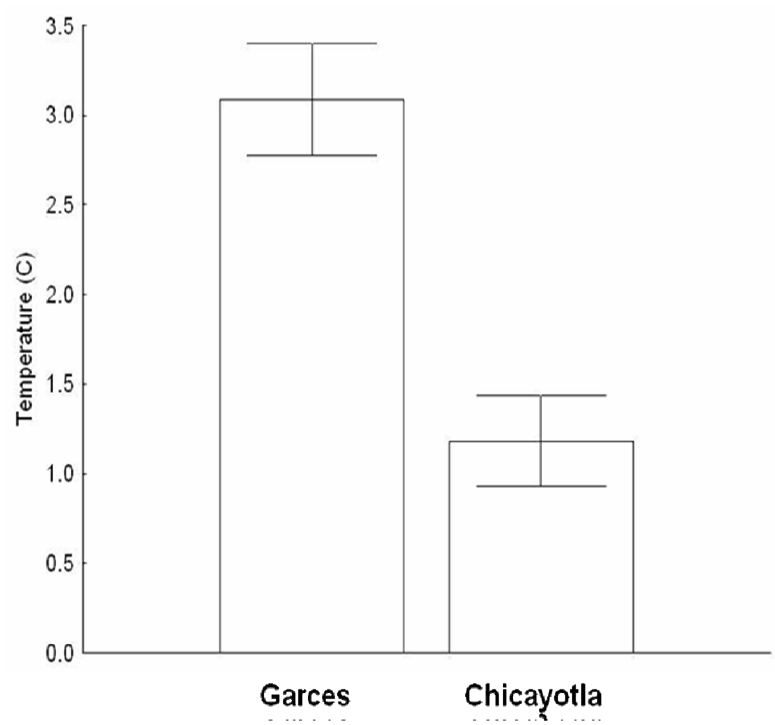

Figure 1. Mean (±SE) daily temperature range (max. temp. - min. temp.) at Garces and Chicayotla between 6/9/06 and 6/15/06. The difference is highly significant ($t_{10} = 4.79$, $P < 0.001$).

Daily and seasonal thermal variation among the Garces, Calnali, and Chicayotla populations contains vital information when investigating tolerance of thermal stress. Loggers recorded seasonal variation between June and December 2007 on the Río Calnali at the Centro de Investigaciones Científicas de las Huastecas "Aguazarca" (CICHAZ). The overall maximum and minimum temperatures recorded were 31.5 and

12.9 degrees Celsius respectively. Daily variation of temperature was recorded for June 9 through June 14 at both Garces and Chicayotla. Results for Garces were as follows: Mean±SE Temp. 29.07 ± 0.17; Minimum temp.: 27.96; Maximum temp.: 31.27; Range: 5.42; Average Daily Temp Range: 3.09. Results for Chicayotla: Mean±SE Temp. 22.70 ± 0.05; Minimum temp.: 21.95; Maximum temp.: 23.77; Range: 1.82; Average Daily Temperature Range: 1.18. (Figure 1) The mean daily temperature variance at Garces showed to be significantly higher than the average daily thermal variation experienced at Chicayotla. This difference exhibits the thermal stress naturally experienced by these two populations on an acute level at the time of collection of the baseline samples.

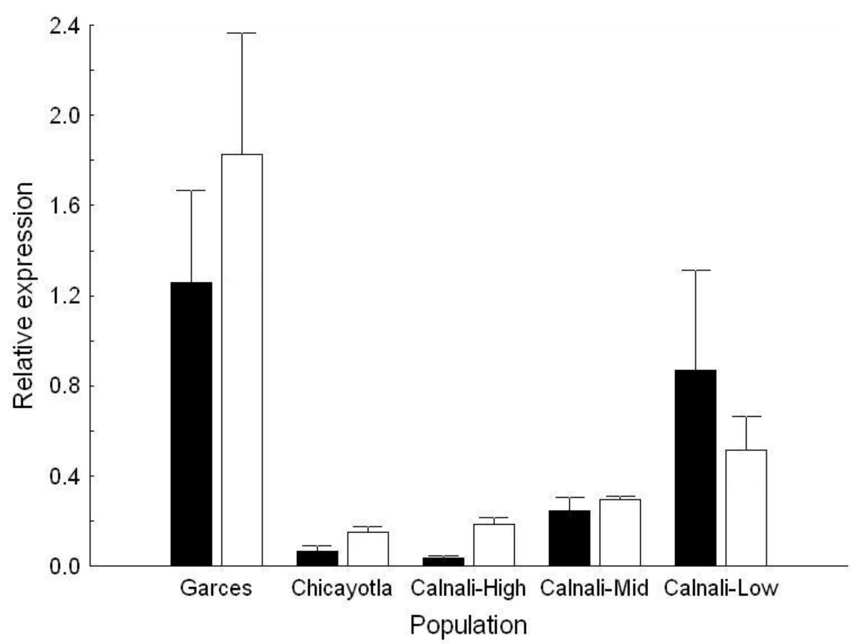

Figure 2. The relative expression levels (mean±SE) of hsp70 (black bars) and hsp90. Within genes, bars with different letters above are significantly different (P<0.05).

(Figure 2) Initial results from the qPCR of the baseline data showed distinctions between the five populations. The hybrid population at Calnali was separated further into Calnali-High, Calnali-Mid, and Calnali-Low for better distinction of results. As initially hypothesized, the expression of both HSP70 and HSP90 were heightened in the Garces population and lowered in the Chicayotla populations, with all three Calnali populations falling in between. This increase in expression of *Hsps* inversely follows the incline of elevation.

After baseline data were collected, results for the acute heat shock experiments became the focus. Differences in reactions to thermal stress were apparent as soon as the acute heat shock experiments were performed on the *X. birchmanni* and *X. malinche* individuals. The four *X. malinche* individuals averaged approximately 10 minutes in the water before loosing equilibrium, withstanding an average maximum temperature of 33.5 degrees Celsius. The four *X. birchmanni* individuals survived the thermal stress longer by obtaining an average time of approximately 14 minutes and loosing equilibrium at an average temperature of 36.75 degrees. Since these eight individuals were immediately sacrificed upon loosing equilibrium, the Hsps data collected from the brain tissue would demonstrate the expression of *Hsps* at the highest level of thermal stress tolerable.

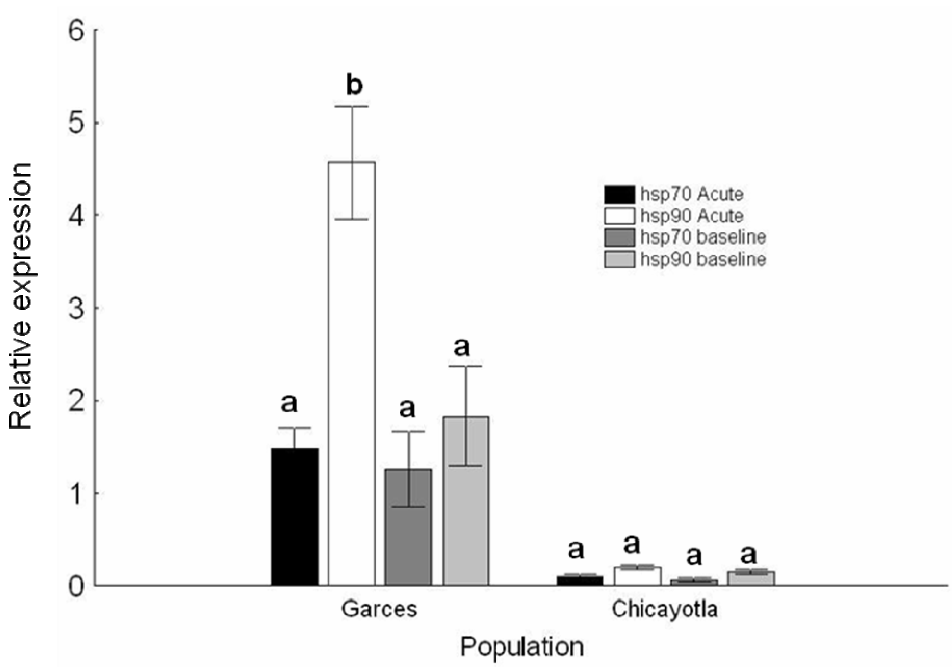

Figure 3. The relative expression levels (mean±SE) of hsp70 following acute thermal stress (black bars), hsp90 following acute thermal stress (white bars), hsp70 baseline (dark grey bars), and hsp90 baseline (light grey bars) for *X. birchmanni* (Garces population) and *X. malinche* (Chicayotla population). Within species, bars with different letters above are significantly different ($P<0.05$).

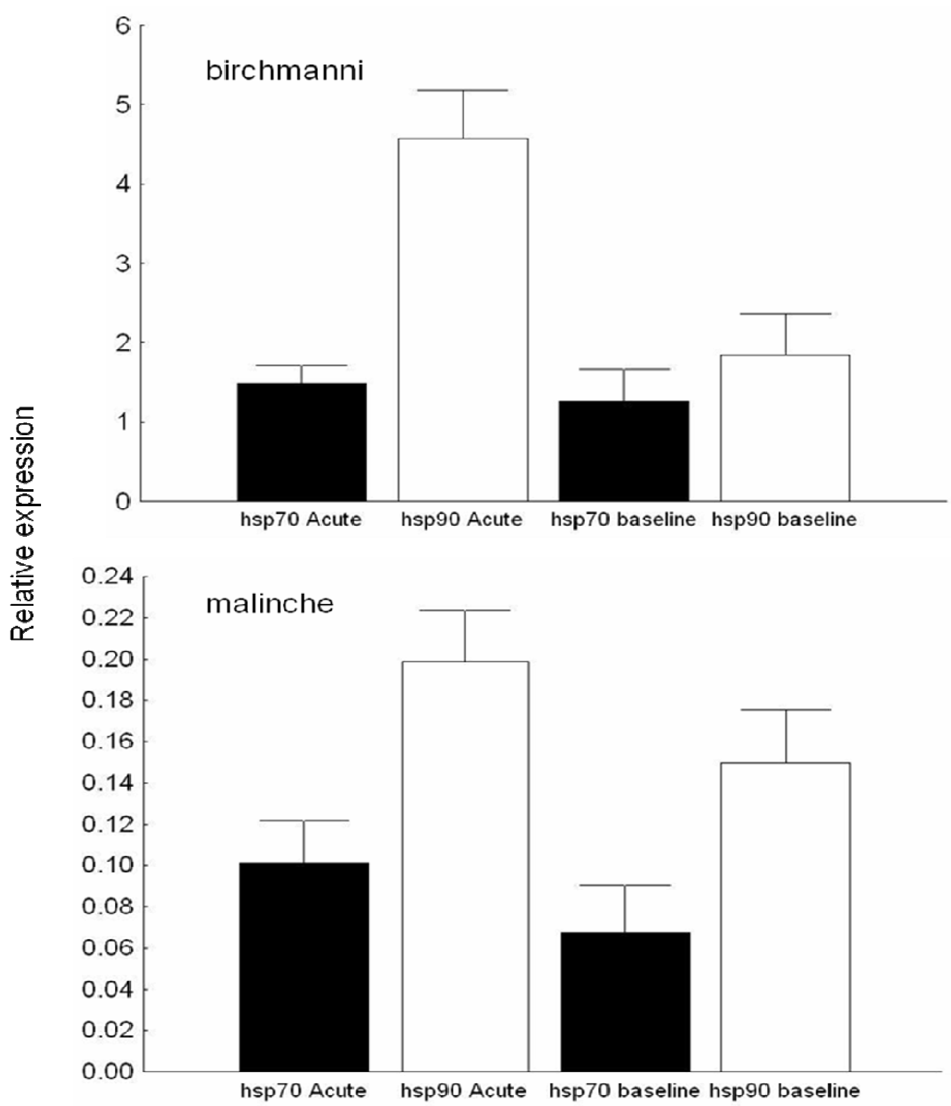

Figure 4. The relative expression levels (mean±SE) of hsp70 for baseline and acute thermal stress (black bars), hsp90 for baseline and acute thermal stress (white bars) for *X. birchmanni* (Garces population) and *X. malinche* (Chicayotla population).

(Figure 3) The qPCR data collected for HSP70 and HPS90 expressed during the acute heat shock experiment shows an increase in Hsps across the board. Examination of *Hsps* expression of the acute heat shock experiment in comparison to baseline *Hsps* expression reveals increased levels of HSP70 and HSP90 in both populations when exposed to acute thermal stress (Figure 4).

CHAPTER IV

DISCUSSION

The protective nature of Hsps is activated whenever a stressor of various types is forced upon an organism. Different Hsps or combination of Hsps are activated based on the type of stress the organism is experiencing. These various Hsps are labeled in accordance to their molecular mass (Tomanek & Somero 1999). The focus of this study lies on the expression of *Hsps* in response to thermal stress, requiring Hsps which are able to tolerate hyperthermia. Although HSP70 and HSP90 both specialize in tolerance of hyperthermia, HSP70 is also able to regulate heat-shock response (Feder & Hofmann 1999). The baseline and acute results demonstrate a higher level of expression in HSP90 than HSP70. The increase in HSP90 expression in both *X. malinche* and *X. birchmanni* after the acute heat shock experiment exhibits the cellular need for HSP90 when experiencing acute thermal stress.

Heat shock response is not simply the cellular response to prolonged exposure to high levels of heat, rather the need of protein chaperones when thermal levels fluctuate frequently or drastically. The raised levels of *Hsps* expression in *X. birchmanni* in comparison to *X. malinche* stem from more drastic daily temperature variance at Garces than Chicayotla. The different environments play a role in daily temperature variance by exposing the Garces population to greater thermal fluctuation as a result of the wider and less covered stretch of river. Sun exposure causes the water to experience a greater variety of temperatures throughout the day than the swift, canopied water of Chicayotla.

Baseline data supports this need for more Hsps in *X. birchmanni* than *X. malinche* by the increased expression of both HSP70 and HSP90 in individuals pulled from the Garces population.

When separating the three hybrid populations at Calnali, there is a significant jump in *Hsps* expression between Calnali-Low and the remaining Calnali populations. This increase in *Hsps* expression correlates the difference in environment that distinguishes the Calnali-Low population from the other Calnali populations. Calnali-Low is located near the base of the town where most of the town waste is released into the river. The pollutants and human waste provide additional environmental stress to the Calnali-Low population while the Calnali-Mid and Calnali-High remain exempt from such pollution. A constantly polluted environment can interfere with an organism's ability to cope with other stressors, such as temperature fluctuations (Fader *et al*. 1994). Therefore, the fish at Calnali-Low must express higher levels of Hsps as protection against the same amount of thermal variance the other Calnali populations experience.

Baseline data shows the relative presence of HSP70 and HSP90 in the hybrid and allotropic populations when the fish are experiencing little to no thermal stress. When individuals were stressed to maximum thermal tolerance in the acute heat shock experiment, the resulting data exposes the maximal amount of HSP70 and HSP90 available for use in hypothermal tolerance. Since individuals from the Garces population are able to use more Hsps when exposed to acute thermal changes, *X. birchmanni* are adapted for greater thermal variance than *X. malinche*.

The results for the acute heat shock experiment give insight in determining if HSP70 and HSP90 expression is the result of genotype or phenotype. All eight individuals used in the acute experiment had experienced constant environmental temperatures for 14 months prior to the experiment. If phenotypic plasticity was responsible for the higher levels of *Hsps* expression in *X. birchmanni* than *X. malinche*, then both species would have equally adapted to the minimal amount of thermal variation experienced during the 14 months in captivity. In actuality, the acute results mimic the baseline data with *X. birchmanni* expressing higher levels of both types of Hsps than *X. malinche*. This suggests that *Hsp* expression may be associated with genetic differences among species, rather than exposure to particular temperature regimes. Testing of this hypothesis through further research on the genotypes of *X. malinche, X. birchmanni* and their hybrids would provide additional understanding of how swordtails are able to vary expression of *Hsps*.

CHAPTER V

CONCLUSIONS

The findings provided in this study conclude that environmental thermal variance among swordtail populations at Chicayotla, Calnali, and Garces results in higher levels of HSP70 and HSP90 in *X. birchmanni* than in *X. malinche. X. birchmanni* tolerate acute changes in temperature more efficiently than *X. malinche* due to higher expression of *Hsps*, cellular chaperones designed to protect vital proteins when exposed to increased levels of stress.

The quick-moving, shaded water at Garces requires little response to thermal variation, while the broader, open water of Calnali and Chicayotla demand higher levels of Hsps to tolerate the increase in daily and seasonal temperature fluctuation. The more *Hsps* an organism can express as a response to stress, the greater resistance the organism has of not succumbing to the added stress. The energy needed to produce Hsps becomes wasteful if an organism does not experience a vast amount of cellular stress. For this reason, there is a correlation between the amount of *Hsps* expressed and the amount of stress endured. A species that endures small amounts of thermal variation is best adapted when expressing small amounts of *Hsps*, while a species with large amounts of thermal variation must express large amounts of *Hsps* to obtain the highest level of fitness for that environment.

The results of this study parallel the correlation of environmental thermal stress and *Hsps* expression. Thermal changes in the environment, such as global warming, will alter which species of swordtails is best fit for the locations of Chicayotla, Calnali, and Garces. Since *X. birchmanni* and *X. malinche* are able to produce hybrids with *Hsps* expression ranging between the two parapatric species, sudden, increased temperatures along the Sierra Madre in Mexico could have drastic affects on the *X. malinche* population found at Chicayotla.

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